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Efficacy and Safety of Frozen Blood for Transfusion in Trauma Patients – A Multi-Center Trial

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14. ABSTRACT
An abbreviated shelf life and storage lesion limit the utility of standard red blood cells (RBCs). Cryopreserved red blood cells (CPRBCs) are frozen from 2-6 days after donation, stored up to 10 years, and washed and deglycerolized prior to use, potentially providing a versatile, fresh, and pure RBC product. We hypothesized that CPRBC transfusion would be equivalent to RBC transfusion in stable trauma patients. We performed a prospective, randomized, double blind study at five level 1 trauma centers. Stable trauma patients requiring transfusion were randomized to old RBCs (>14 storage days), young RBCs (<14 storage days), or CPRBCs. Tissue oxygenation and biochemical and inflammatory parameters were measured and clinical outcomes were determined. In total, 256 patients were randomized (84 young, 86 old, and 86 CPRBCs). The patients were well matched for injury severity and demographics ($p>0.2$). Pre-transfusion and final hematocrits were similar ($p>0.68$). Patients randomized to CPRBCs required two units, compared to four units of RBCs in the other groups ($p<0.001$). Transfusion of old RBCs resulted in reduced tissue oxygenation, while transfusion of CPRBCs resulted in an increase ($p<0.05$). CPRBCs contained significantly less α2-macrogobulin, haptoglobin, c-reactive protein, serum amyloid P, and free hemoglobin than the other groups ($p<0.001$). Interleukin-2 was elevated in patients who received CPRBCs compared to old RBCs. There was no difference in organ failure, infection rate, or mortality between the three groups ($p>0.22$). Transfusion of CPRBCs is at least as safe and effective as transfusion of RBCs and results in a reduced transfusion requirement.

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1.0 SUMMARY

An abbreviated shelf life and storage lesion limit the utility of standard red blood cells (RBCs). Cryopreserved red blood cells (CPRBCs) are frozen from 2-6 days after donation, stored up to 10 years, and washed and deglycerolized prior to use, potentially providing a versatile, fresh, and pure RBC product. We hypothesized that CPRBC transfusion would be equivalent to RBC transfusion in stable trauma patients. We performed a prospective, randomized, double blind study at five level 1 trauma centers. Stable trauma patients requiring transfusion were randomized to old RBCs (>14 storage days), young RBCs (<14 storage days), or CPRBCs. Tissue oxygenation and biochemical and inflammatory parameters were measured and clinical outcomes were determined. In total, 256 patients were randomized (84 young, 86 old, and 86 CPRBCs). The patients were well matched for injury severity and demographics ($p>0.2$). Pre-transfusion and final hematocrits were similar ($p>0.68$). Patients randomized to CPRBCs required two units, compared to four units of RBCs in the other groups ($p<0.001$). Transfusion of old RBCs resulted in reduced tissue oxygenation, while transfusion of CPRBCs resulted in an increase ($p<0.05$). CPRBCs contained significantly less α2-macrogobulin, haptoglobin, C-reactive protein, serum amyloid P, and free hemoglobin than the other groups ($p<0.001$). Interleukin-2 was elevated in patients who received CPRBCs compared to old RBCs. There was no difference in organ failure, infection rate, or mortality between the three groups ($p>0.22$). Transfusion of CPRBCs is at least as safe and effective as transfusion of RBCs and results in a reduced transfusion requirement.

2.0 INTRODUCTION AND BACKGROUND

2.1 Red Blood Cell Transfusion in the Trauma Critical Care Setting

Approximately 15% of blood transfused in the United States is given to trauma patients [1,2]. Anemia is a common problem in critically ill adults, and it has important prognostic implications for morbidity and mortality [1-3]. Several studies have documented the prevalence of anemia in critically ill patients, as well as the high rate of blood transfusions administered in the intensive care unit (ICU) [3]. Critically ill patients lose from 20 to 40 mL of blood daily through phlebotomy, and patients with indwelling arterial catheters lose approximately 900 mL of blood during their stay in the ICU [3-7]. Red blood cell (RBC) production in these patients is often reduced and they are not able to recover from these losses. Even healthy volunteers undergoing phlebotomy for blood donation can only regenerate about 10 mL of RBCs per day. As a consequence of anemia, most critically ill patients receive liquid preserved RBC (LPRBC) transfusions [3,5].

Blood transfusion practices remain controversial and generate a tremendous amount of research and debate. Ideally, best practice would be based on well-founded clinical indications for LPRBC transfusion. The conclusion of the 1988 National Institutes of Health Consensus Conference is still of great importance today: no single measurement can replace good clinical judgment concerning the need for red cell transfusion [8]. Moreover, defining such indications is essential to avoid unnecessary transfusions and to ensure that blood is not withheld when it may be of benefit. There are several well-accepted reasons to administer a packed red blood cell (PRBC) transfusion based on the physiologic properties of red cells. These include restoration

and maintenance of intravascular volume, activation of platelet-driven hemostasis, restoration of tissue perfusion, and prevention of the development of organ failure and death [9-13].

2.1.1 Blood Transfusion Triggers. No prospective, randomized clinical trials comparing LPRBC transfusion with placebo have been conducted in the past, and none are likely to be performed. Only experimental and clinical data have shown the positive effects of appropriate transfusion [5,8,13-16]. Kitchens performed a review of surgical outcomes in Jehovah's Witness patients who underwent a major operation without blood transfusion [17]. Mortality associated with anemia occurred in 1.4%. In another study, morbidity occurred in 9.4% of patients with very low hemoglobin (Hb) who refused transfusion. The odds of death in patients with a postoperative Hb level ≤ 8 g/dL increased 2.5 times for each gram decrease in Hb level [18].

Level 1 data addressing the optimal transfusion threshold are based on a randomized controlled trial by Hébert et al. that showed that critically ill patients who are not actively bleeding should not receive blood until their Hb is < 7 g/dL [19]. A meta-analysis of published data available in 2010 showed that the use of a restrictive transfusion trigger resulted in an average savings of one unit of PRBCs per transfused patient, but the reduction in use of allogeneic blood did not decrease 30-day mortality [20]. Recently published retrospective data in patients with severe isolated traumatic brain injury have confirmed the safety of a restrictive transfusion practice [21,22].

2.1.2 PRBC and Mortality. Blood transfusion and PRBC storage duration have been identified as potential causes of increased morbidity and mortality in several studies [23-28]. In a large study, Malone et al. assessed the effect of blood transfusion on outcome in 15,534 trauma patients over 3 years [29]. After controlling for all possible confounding variables, they found that LPRBC transfusion was a strong independent predictor of mortality. Another study published by Murrell et al. did not find an association between the dose of aged blood and mortality in major trauma patients [30]. Van de Watering et al. reported an analysis of 2,732 coronary artery bypass patients that showed an association between storage time and survival [31]. Koch et al. retrospectively studied 6,002 cardiac surgery patients, comparing those who received blood <14 days old with those who received blood >14 days old [25,32]. In this study, there was a significantly increased mortality in patients who were transfused with older blood. The presence of numerous confounders with inadequate stratification has been addressed in several letters to the editor of the *New England Journal of Medicine*, as well as discussions in other journals [33-36]. Weinberg et al. analyzed the number of LPRBCs that were <14 days old and >14 days old in 1,813 transfused trauma patients [26]. Both blood transfusion and age of blood were associated with increased mortality. Similar to other retrospective studies, baseline variables were not controlled for and there was an arbitrary stratification of the number of units transfused.

2.1.3 LPRBC and Infectious Complications. Numerous studies have shown that one of the most frequent causes of morbidity and mortality after traumatic injuries is development of infections [37-42]. These studies reveal an association between blood transfusion and a higher incidence of infections in trauma patients [37,39-41,43,44]. Offner et al. demonstrated increased risk of infection in critically injured patients transfused blood that was older than either 14 days or 21 days compared to a matched group that received younger blood [37]. However, there was no association between blood age and the occurrence of sepsis in the study by Gajic et al. [45].

Similarly, Yap et al. found no relationship between postoperative pneumonia and mean storage time, oldest PRBC unit transfused, or storage time >30 days [42].

2.1.4 LPRBC and Organ Failure. Blood transfusion has consistently been shown to be a major risk factor for post-injury multiple organ failure [24,26,27,29,46,47]. Koch et al. found associations between age of blood transfused and the occurrence of respiratory failure, renal failure, and multiple organ failure [25]. Weinberg et al. recently published an analysis of the impact of blood age on trauma patients requiring ICU admission [48]. They showed that transfusion of LPRBCs stored >14 days was associated with renal failure and pneumonia. The odds ratios were adjusted for age, sex, injury severity, thoracic injury, mechanical ventilation, and transfusion volume.

2.2 RBC Storage Lesion

RBCs have a definite lifespan that is well regulated in spite of their lack of a nucleus and capacity for protein synthesis [49]. Donated blood is a mixture of cells with different ages. During preservation under standard blood bank conditions, these donor RBCs undergo a series of reversible and irreversible, functional, and structural changes, which are often collectively referred to as a "storage lesion." The storage lesion manifests with various speeds as the age of the cells differs. The storage lesion has been well documented for decades, but our understanding of the mechanisms involved in these changes and their clinical consequences remains incomplete [50-54]. The storage lesion elements are described in the following sections.

2.2.1 Loss of Nitric Oxide (NO) Bioactivity. Storage of blood leads to a rapid loss in NO bioactivity, which has been explained by a decline in S-nitrosohemoglobin (SNO-Hb) [55-57]. Donated blood is stored in an acidic-buffered solution, which may accelerate SNO-Hb decay. Transfusion of stored blood is associated with a decreased ability of RBCs to affect hypoxic vasodilatation. SNO-Hb can be replenished, restoring its physiologic effects [57].

2.2.2 Depletion of 2,3-Diphosphoglycerate (2,3-DPG). 2,3-DPG exists in high concentrations in RBCs and it is the major allosteric modifier of oxygen affinity. It facilitates oxygen delivery to the tissues [58]. Its level in LPRBCs progressively declines during storage, and after 2 weeks it is completely depleted, resulting in a shift of the oxygen dissociation curve to the left and subsequent impaired oxygen delivery [55]. De novo synthesis of 2,3-DPG occurs after transfusion; however, the speed of restoration varies and can take up to 72 hours [59]. Based on these observations, it has been speculated that transfusion of large amounts of stored PRBCs may have an adverse clinical influence on oxygen delivery [52,54]. Studies in baboons and rats do not support a key role for 2,3-DPG in the off-loading of oxygen [60,61].

2.2.3 Morphology, Deformability, and Viability. During storage, RBCs undergo a predictable change in morphology, evolving from deformable biconcave disks to reversibly deformed echinocytes, and then to irreversibly deformed spheroechinocytes [50,61,62]. These shape changes in stored blood correlate with decreased RBC survival as a result of increased splenic sequestration and destruction [63-67]. The decreased deformability of RBCs may contribute to impaired perfusion and oxygen delivery in peripheral tissues, and rigid cells might directly block capillaries [61,68,69]. However, using fluorescent RBC labeling, Parthasarathi et al. showed that

reduced RBC deformability leads to a shunting of RBCs through larger diameter vessels in the microcirculation, suggesting that stiff RBCs are not as likely to obstruct vessel flow as previously thought [68]. The clinical impacts of these changes are unknown. Langenfeld et al. demonstrated that trauma patients had decreased erythrocyte deformability [70]. There is a positive correlation between RBC transfusion and RBC deformability in critically ill patients [71]. We can logically suppose that the transfusion of stored RBCs may have adverse effects on microcirculatory flow and oxygen utilization, particularly in vulnerable patients.

2.2.4 RBC Hemolysis. There is a strong relationship between blood age and the levels of free Hb in PRBCs primarily due to hemolysis [72-74]. Free Hb levels have been shown to increase from 17.4 mg/dL at 2 days to 90.2 mg/dL at 26 days in stored PRBCs [72,75]. Free Hb plays an important role in the disruption of normal NO physiology by rapidly scavenging NO, producing vasoconstriction and decreasing oxygen delivery to tissues [76,77]. Free Hb is capable of scavenging endothelial-derived NO much more efficiently than RBC-encapsulated Hb [78-81]. The causal relationship between excess free Hb in the bloodstream, pulmonary and systemic hypertension, decreased organ perfusion, and increased mortality was found in clinical trials of Hb-based blood substitutes [77]. In addition, free Hb also exerts direct cytotoxic, inflammatory, and pro-oxidant effects that adversely impair endothelial function [82,83]. The most important protective mechanism against the destructive effects of free Hb is its binding by the serum protein haptoglobin. This is limited by the concentration of free Hb and the rate of haptoglobin synthesis.

2.2.5 Residual Leukocytes and Platelets during Storage. It is commonly assumed that PRBCs contain only erythrocytes; in fact, pure blood products are rarely clinically achievable and even leukoreduced PRBCs contain a mixture of different blood cell components [84]. Residual leukocytes and platelets in PRBCs can cause the accumulation of various soluble bioactive substances during storage [53,83]. Histamine, complement, lipids, lactate, free Hb, and cytokines can be detected in the supernatant of stored non-leukocyte and platelet-reduced PRBCs [85-88]. Transfusion of stored PRBCs triggers neutrophil activation and the release of interleukin-8 (IL-8), as well as secretory phospholipase A_2, thereby predisposing recipients to systemic inflammatory response syndrome [89-91]. Furthermore, in animal studies plasma from stored red cells causes vasoconstriction and lung injury [92].

2.3 Cryopreserved Packed Red Blood Cells (CPRBCs)

2.3.1 Biochemical and Functional Characteristics of CPRBCs. Published data have demonstrated that RBCs frozen by any of the accepted methods have comparable functional properties after thawing and deglycerolization as they possessed prior to freezing. The primary function of RBCs is the transport of oxygen to the tissue [7,27,93,94]. O'Brien and Watkins showed that the oxygen-Hb dissociation curve of frozen-thawed-washed RBCs was similar to that of fresh blood collected in standard anticoagulant [95]. 2,3-DPG levels are comparable to pre-freezing levels and are only affected by the pre-storage interval before freezing [96]. There are concordant results that all glycerolization and deglycerolization procedures with or without freezing result in a normal concentration of adenosine triphosphate in RBCs, hemolysis less than 1%, and RBC potassium levels within 60% of normal [96-98]. Cryopreservation appears to prevent storage membrane changes. RBC membrane phosphatidylserine externalization, cluster

of differentiation 47 expression, and microvesiculation have been shown to be the same prior to freezing, immediately after thawing, and in hypothermic storage control specimens [99]. These data suggest that the freeze-thaw process prevents the development of the storage lesion [99].

2.3.2 Advantage of a Post-Thaw Wash Step. RBCs frozen with glycerol and stored at -80°C are washed after thawing to reduce the glycerol level to <1% [100,101]. This step is beneficial by removing glycerol, plasma, and non-plasma biologically active substances that may have physiologic significance by playing a role in inflammation, hemostasis, vascular dysfunction, and transfusion reactions [102,103]. Indeed, data from our recently published single-center study [104] show that CPRBCs have dramatically lower levels of bioactive proteins that inhibit RBC function and O_2 delivery, which is likely due to the post-thaw washing process. Washing the RBCs reduces these substances to <5% of their original value [105-107]. Exposure of thawed cells to the first saline wash induces an additional 1% hemolysis. This may be a selective hemolysis of "subhemolytically" damaged or senescent cells. "Subhemolytic" damage due to freezing has been shown by Rowe et al. to be related to *in vivo* erythrocyte age, the older cells being more susceptible [108]. These damaged cells probably would not survive after transfusion. The excellent *in vivo* survival indicates that more fragile cells are removed during the post-thaw processing [109,110]. This wash phase also provides some degree of clinical flexibility not found in a conventional stored PRBC unit [111]. The suspension medium of the frozen RBCs can be adjusted to meet the needs of patients. Although RBCs can be resuspended in isotonic solution achieving high hematocrit (90%), the viscosity is low and the deglycerolized RBCs are easier to administer than PRBCs or whole blood [112].

Cryopreservation, which enables prolonged storage of RBCs, is designed to maximize RBC recovery, yet it is not optimal for leukocytes and platelets [100,113-122]. It has been subsequently shown that both glycerolization and freezing destroy white blood cells (WBCs), particularly the granulocytes [123,124]. These damaged cells aggregate during freeze-thaw-wash processing and are absent from the final washed suspension, usually remaining in the processing equipment. The previously freeze-thaw-washed RBCs become leukoreduced [105-107,125]. The deglycerolization process can bring the WBC count below the 5 × 108 WBC threshold that is effective in reducing transfusion reactions [126].

2.3.3 Clinical Use of CPRBCs. There has been extensive use of CPRBCs in civilian and military practice, especially during the period from 1960-1980 [117,127-131]. In September 1987, the Food and Drug Administration approved a 10-year storage period for glycerol-frozen RBCs maintained at -80°C, and according to the American Association of Blood Banks standard, the blood should be frozen within 6 days of collection [132,133]. There are no previous randomized clinical studies comparing the transfusion of cryopreserved allogeneic RBCs with hypothermic-preserved liquid RBCs. Data on *in vivo* functionality and outcomes after transfusion with CPRBCs are limited. The first clinical study investigating the safety and efficacy of the allogeneic transfusion of CPRBCs was conducted at the U.S. Navy hospital at Danang, South Vietnam [131]. Post-transfusion plasma Hb, bilirubin, platelets counts, and serum creatinine levels were not different between patients transfused with CPRBCs or LPRBCs [131].

A component transfusion therapy program utilizing CPRBCs has been developed at Cook County Hospital in Chicago [127]. CPRBCs have been used for up to 64% of transfusions, depending on the availability of erythrocytes for freezing. The institution of frozen blood was associated with a decrease in the incidence of transfusion reactions from 0.57% to 0.11% [127].

O'Brien et al. reported the use of CPRBCs in vascular surgery and in extracorporeal circulation [128]. Thawed, deglycerolized RBCs, which had been resuspended in thawed heparinized plasma, after prolonged circulation through a pump oxygenator, showed no significant changes in properties as compared to recirculated, fresh heparinized blood [128].

CPRBCs were used in many U.S. centers in the 1960s and 1970s for medical and logistical reasons, but due to concerns about cost, their delayed availability, and short half-life after thawing, their use has diminished [116,118,119,127,128,130,134,135]. The ACP® 215 (Haemonetics Corp., Braintree, MA) has simplified and accelerated the thawing process, and thawed RBCs can now be stored for up to 14 days. In addition to the theoretical benefits of avoiding the storage lesion and the benefits of post-thaw washing, the use of CPRBCs may also result in decreased cost by avoiding waste seen with stored LPRBCs.

3.0 MATERIALS AND METHODS

This was a prospective, randomized, multi-center, double blinded study. Institutional Review Board approval was obtained at the following clinical sites: Oregon Health & Science University, University of Cincinnati, University of Texas Health Science Center at Houston, University of Texas Health Science Center at San Antonio, and University of Texas Southwestern Medical Center. Patients admitted to the participating trauma centers with an Injury Severity Score (ISS) > 4 and the potential need for a blood transfusion were eligible. Patients who had received a massive transfusion (≥10 units RBCs in 24 hours) within the last 3 months or a transfusion within the previous 24 hours, were pregnant, or were less than 15 years old were excluded. Patients needing emergent interventions were not included due to processing time. The thawing and deglycerolization process takes approximately 2 hours. In addition, patients with bilateral upper extremity injuries preventing the placement of a tissue oxygenation (StO_2) monitor on the thenar eminence were excluded. Consent was obtained from the patient or designated medical representative. Enrolled patients were blindly randomized to one of three groups: LPRBCs ≤ 14 days old, LPRBCs > 14 days old, or CPRBCs. Patient demographic information (gender, age, ISS, Acute Physiology and Chronic Health Evaluation II score) and clinical outcomes (length of stay, acute respiratory distress syndrome, acute renal failure, transfusion reaction, deep venous thrombosis, and mortality) were recorded. Acute respiratory distress syndrome was defined as outlined by the American-European Consensus Conference. Acute renal failure was assessed using the RIFLE [**R**isk, **I**njury, **F**ailure, **L**oss of kidney function, and **E**nd-stage kidney disease] classification as outlined by the Acute Dialysis Quality Initiative workgroup.

All decisions to administer PRBCs were at the discretion of the primary treatment team. The general threshold for transfusion is hemoglobin less than 7 g/dL except for patients with severe head injury, in whom the threshold is hemoglobin less than 8 g/dL. Thawing and deglycerolization were performed once the order for PRBCs was placed. Units were transfused within 3 days of thawing. Post-thaw, cells were preserved in AS-3 solution at 4°C. While the Food and Drug Administration guidelines allow use of cryopreserved RBCs up to 14 days after thawing, all units were transfused within 72 hours to minimize confounding comparisons.

The method of cross matching varied depending on the patient's antibody screen results. Antiglobulin, a serological test, was performed by the Gel microtube methods when the patient's serum was found to be without antibodies. When no antibodies were present and there were no discrepancies in the ABO and Rh type, an electronic cross match was performed. When no

antibodies were present and the patient was otherwise ineligible for computer cross match, an immediate spin serological test was performed.

Once a transfusion order was placed for an enrolled patient, a near infrared spectroscopy device (InSpectra™ StO_2 Oxygenation Monitor, Hutchinson Technology, Hutchinson, MN) was placed an hour prior to the start of transfusion. StO_2 was measured continuously up to 12 hours after completion of the transfusion. This device uses a thenar eminence sensor pad to assess StO_2. In patients with radial arterial lines or those with upper extremity injuries, the sensor was placed on the other hand. Near infrared spectroscopy is a previously validated monitoring system that measures tissue perfusion noninvasively using near infrared light to assess the ratio of oxygenated hemoglobin to total hemoglobin in the underlying tissue.

Before initiation and after completion of each transfusion, 10-cc blood samples were obtained from the subjects. Halfway through the transfusion, 10 cc of blood was obtained from the LPRBC and CPRBC units. Final laboratory samples were obtained from the subjects 12 hours after completion of the last transfused unit. Based on data showing the association between blood cell transfusion and venous thromboembolism, thrombelastography (TEG) was performed on all subject samples to evaluate coagulation status. A complete blood count was also performed at baseline, post-infusion, and 12 hours. All samples were additionally assessed for biochemical changes. Hemoglobin was analyzed by enzyme-linked immunosorbent assay (Bethyl Laboratories, Montgomery, TX). Haptoglobin, serum amyloid P (SAP), and C-reactive protein (CRP) were evaluated utilizing the Bio-Plex Pro Human Acute Phase 4-Plex Panel (Bio-Rad Laboratories Inc., Hercules, CA). SAP and CRP were studied due to their known anti-coagulation effects. Elevated levels of SAP, in the presence of heparin, can cause a hypocoagulable state. At supra-physiologic levels, SAP's anti-coagulation effects manifest in the absence of heparin. CRP, a SAP homologue, also retards coagulation. Finally, hemolysis during storage could be assessed through changes in haptoglobin, a free hemoglobin scavenger.

2,3-DPG levels were quantified with a commercially available kit (Roche Diagnostics, Indianapolis, IN). Cytokines [IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GMCSF), tumor necrosis factor alpha (TNFα), and interferon gamma (INFγ)] were measured using the Human Cytokine 8-Plex Assay (Bio-Rad Laboratories Inc., Hercules, CA). Coagulation data (prothrombin time, partial thromboplastin time, D-dimer, and fibrinogen) were measured using standard tests (Diagnostica Stago Inc., Parsippany, NJ). TEG data were obtained using a TEG 5000 Thrombelastograph Hemostasis Analyzer System (Haemonetics, Braintree, MA). Citrated whole blood was placed in kaolin-activated cups to do these assessments. TEG, a measure of clot formation efficiency, is characterized by four parameters: R, time to onset of clotting; alpha angle, rate of fibrin cross-linking; MA, maximum amplitude or clot strength; and LY30, lysis 30 minutes after MA.

All data were analyzed using SPSS (IBM, Armonk, NY). All continuous normally distributed data were analyzed using Student's t-tests. All non-parametric data were analyzed using a Mann-Whitney U test.

4.0 RESULTS

In total, 256 trauma patients were enrolled and received at least one unit of PRBCs; 76 received LPRBCs ≤14 days, 73 received LPRBCs >14 days, and 82 received CPRBCs. Fifty-two percent of patients (134) received two units compared to 48% (122) who received one unit during observation. The three groups were well matched for age, gender, and ISS scores (Table 1). The CPRBC group received fewer units of RBCs overall compared to both LPRBC groups ($p<0.00$). There was no difference in the median transfusion time for each observation.

Table 1. Demographic Information

Demographic	Old LPRBCs Median (IQR) (n=86)	Young LPRBCs Median (IQR) (n=82)	CPRBCs Median (IQR) (n=86)	p-value[a]
Age (yr)	47.9 (28.1, 67.0)	52.3 (29.4, 62.5)	49.3 (30.9, 62.6)	0.89
Gender (male/female)	40/46	32/50	29/57	0.27
ISS	19 (11, 26)	19 (10, 32)	19 (14, 29)	0.97
Units Transfused	4 (2, 6)	4 (2, 6)	2 (1, 4)[b]	0.00
Transfusion Time (min)	75 (40.5, 130)	90 (51.25, 141.25)	86 (37.75, 127.25)	0.60
Age of Blood (d)	32 (23, 36)[c]	7.5 (5, 11)	3 (1, 3)	0.00

IQR = interquartile range.
[a]p-value between groups.
[b]CPRBCs < Old LPRBCs and Young LPRBCs.
[c]Old LPRBCs > Young LPRBCs and CPRBCs.

4.1 Tissue Oxygenation

To avoid minute-to-minute variations, which are characteristics of StO_2 measurements, and to better characterize the trends, an area under the curve (AUC) analysis was performed. Mean values for StO_2 tracings were compared in patients receiving CPRBCs and LPRBCs (Table 2, Figure 1). All data were normalized to baseline values (-60 to 0 minutes). The CPRBC group was noted to be significantly higher 240 to 300 minutes after start of transfusion (0 minute) compared to the Old LPRBC group ($p=0.03$). No other differences were noted between the three groups.

Differences were noted in the Old LPRBC group with StO_2 values lower 120 minutes through 300 minutes after the start of transfusion when compared to baseline. The CPRBC group was significantly higher in the first hour of transfusion compared to baseline. There were not any differences over time noted in the Young LPRBC group.

4.2 Coagulation Assays

Median values of standard coagulation parameters of international normalized ratio, activated partial thromboplastin time, fibrinogen, and D-dimer are represented in Table 3. No differences are noted between groups or over time for these parameters. Additionally, there were no differences observed in TEG parameters between groups or over time (Table 4).

As would be expected, hematocrit values were significantly higher in all groups after each unit transfused and at 12-hours post transfusion. Platelet counts were lower in the CPRBC group after each unit transfusion. However, overall, the CPRBC group had a higher platelet count that was significantly different after the first unit and 12-hours post transfusion. These differences were not clinically significant (Table 5).

Table 2. Tissue Oxygenation AUC

Time (min)	Old LPRBCs		Young LPRBCs		CPRBCs		Overall p-value[a]
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	
AUC, -60 to 0	73	6000.0±0	76	6000.0±0	82	6000.0±0	--
AUC, 0 to 60	73	6004.0±521.6	76	6052.0±276.0	82	6079.7±281.3[b]	0.45
AUC, 60 to 120	73	5917.3±473.2	73	5983.4±332.4	81	5964.45±355.0	0.57
AUC, 120 to 180	72	5892.1±451.9[b]	72	6019.8±349.0	79	6000.8±455.0	0.15
AUC, 180 to 240	71	5880.7±432.8[b]	72	5976.9±447.4	79	6018.6±511.8	0.19
AUC, 240 to 300	71	5845.4±492.6[b]	71	6014.8±490.0	79	6056.1±527.2[c]	0.03
AUC, 300 to 360	61	5882.5±584.6	70	6001.7±574.2	79	6038.0±538.5	0.25

SD = standard deviation.
Note: Results normalized to baseline (-60 to 0 minutes).
[a]p-value (over time) >0.05.
[b]Old LPRBCs decrease over time; CPRBCs increase in the first hour of transfusion.
p-value (over time) <0.05 vs. baseline.
[c]CPRBCs > Old LPRBCs at 240 to 300 min.

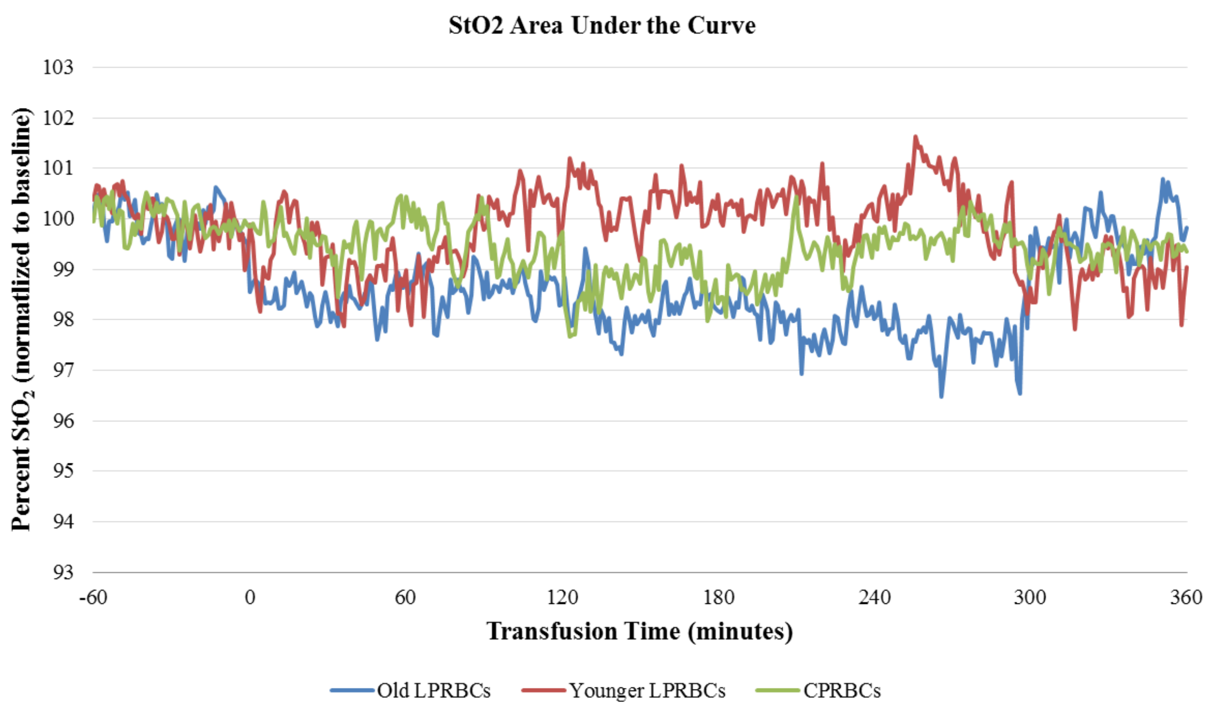

Figure 1. Tissue oxygenation.

Table 3. Standard Coagulation Parameters

Time Point	Old LPRBCs		Young LPRBCs		CPRBCs		p-value[a]
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
INR[b]							
Baseline	75	1.1 (1.1-1.2)	65	1.1 (1.00-1.3)	69	1.1 (1.0-1.2)	0.20
Unit 1	67	1.1 (1.0-1.2)	63	1.1 (1.00-1.2)	67	1.1 (1.0-1.2)	0.72
Unit 2	37	1.1 (1.0-1.1)	36	1.1 (0.98-1.2)	29	1.1 (1.0-1.2)	0.59
12 h post	72	1.1 (1.0-1.2)	62	1.1 (1.00-1.2)	65	1.1 (1.0-1.2)	0.87
aPTT[c] (s)							
Baseline	75	35.6 (30.30-40.7)	65	33.9 (29.8-39.1)	69	33.1 (29.1-41.6)	0.64
Unit 1	68	35.6 (29.93-40.3)	61	33.0 (29.6-38.4)	67	33.2 (29.2-41.5)	0.50
Unit 2	37	32.6 (27.80-38.2)	36	33.3 (29.6-38.4)	29	32.6 (29.2-35.5)	0.81
12 h post	70	35.5 (30.30-41.2)	62	33.3 (29.8-40.1)	64	35.4 (30.1-42.2)	0.78
Fibrinogen[d] (mg/dL)							
Baseline	74	535 (414-687)	65	574 (428-724)	69	618 (483-727)	0.19
Unit 1	68	585 (453-704)	62	575 (456-716)	67	634 (484-745)	0.53
Unit 2	37	564 (437-695)	36	564 (401-680)	29	564 (412-663)	0.82
12 h post	71	576 (442-704)	62	621 (465-769)	64	624 (467-749)	0.31
D-dimer[e] (mg/mL)							
Baseline	70	3.94 (2.30-7.40)	61	3.9 (2.7-8.6)	68	4.2 (2.3-7.2)	0.89
Unit 1	68	4.18 (2.69-7.47)	58	4.4 (2.8-9.4)	65	3.8 (2.6-6.8)	0.52
Unit 2	35	3.09 (1.83-4.86)	36	5.9 (2.8-9.4)	27	4.5 (2.6-7.9)	0.09
12 h post	65	3.92 (2.64-7.30)	57	5.0 (2.9-8.7)	61	4.3 (3.2-8.8)	0.37

IQR = interquartile range.
[a]p-value between groups.
[b]p-value (over time): Old LPRBCs = 0.24; Young LPRBCs = 0.49; CPRBCs = 0.56.
[c]p-value (over time): Old LPRBCs = 0.49; Young LPRBCs = 0.90; CPRBCs = 0.62.
[d]p-value (over time): Old LPRBCs = 0.79; Young LPRBCs = 0.36; CPRBCs = 0.50.
[e]p-value (over time): Old LPRBCs = 0.35; Young LPRBCs = 0.81; CPRBCs = 0.29.

Table 4. Comparison of TEG Parameters between Groups and Over Time

Time Point	Old LPRBCs		Young LPRBCs		CPRBCs		p-value[a]
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
R[b] (min)							
Baseline	71	5.2 (4.0-6.5)	69	5.1 (4.1-6.4)	76	4.7 (3.6-6.2)	0.31
Unit 1	65	5.1 (3.7-6.2)	64	4.7 (3.8-6.5)	66	5.2 (4.0-7.0)	0.83
Unit 2	36	4.1 (3.2-5.5)	37	4.8 (3.5-6.0)	26	4.4 (3.4-6.2)	0.78
12 h post	72	5.3 (3.8-7.5)	63	4.9 (3.6-6.7)	67	5.4 (4.2-6.7)	0.54
alpha[c] (deg)							
Baseline	71	74.4 (69.7-77.3)	69	75.3 (70.7-77.8)	76	76.3 (69.2-79.0)	0.33
Unit 1	65	73.1 (68.4-77.2)	64	74.1 (69.2-77.2)	66	75.4 (68.6-78.3)	0.38
Unit 2	36	74.5 (67.8-77.6)	37	70.0 (64.5-77.5)	26	75.6 (68.4-79.9)	0.29
12 h post	72	71.9 (65.2-77.5)	63	73.6 (67.2-77.2)	67	74.2 (68.7-78.4)	0.35
MA[d] (mm)							
Baseline	71	71.7 (65.2-77.9)	69	75.3 (70.7-77.8)	76	73.8 (67.7-79.9)	0.26
Unit 1	65	71.6 (64.3-75.8)	64	74.1 (69.2-77.2)	66	73.2 (68.3-80.8)	0.10
Unit 2	36	69.8 (63.4-75.5)	37	72.8 (68.4-75.9)	26	71.3 (65.7-80.8)	0.44
12 h post	72	72.4 (65.3-77.2)	63	73.2 (67.8-77.3)	67	72.7 (66.6-78.2)	0.68
LY30[e]							
Baseline	68	0.6 (0.1-2.0)	67	0.4 (0.0-1.5)	75	0.7 (0.0-1.9)	0.42
Unit 1	62	0.5 (0.1-1.9)	62	0.3 (0.0-1.2)	63	0.5 (0.0-2.2)	0.41
Unit 2	35	0.9 (0.1-2.2)	37	0.5 (0.0-1.5)	25	0.8 (0.1-1.7)	0.52
12 h post	71	0.4 (0.0-1.6)	62	0.3 (0.0-1.1)	65	0.5 (0.0-1.3)	0.40

R = time to onset of clotting; alpha = rate of fibrin cross-linking; MA = maximum amplitude or clot strength; LY30 = lysis at 30 min after MA.

[a]p-value between groups.

[b]p-value (over time): Old LPRBCs = 0.12; Young LPRBCs = 0.60; CPRBCs = 0.25.

[c]p-value (over time): Old LPRBCs = 0.55; Young LPRBCs = 0.59; CPRBCs = 0.81.

[d]p-value (over time): Old LPRBCs = 0.51; Young LPRBCs = 0.54; CPRBCs = 0.82.

[e]p-value (over time): Old LPRBCs = 0.55; Young LPRBCs = 0.75; CPRBCs = 0.87.

Table 5. Comparison of Hematocrit and Platelet Counts between Groups and Over Time

Time Point	Old LPRBCs		Young LPRBCs		CPRBCs		p-value[a]
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	
Hematocrit[b]							
Baseline	80	20.88±2.21	75	21.14±2.0	81	20.88±2.23	0.68
Unit 1	74	23.30±2.10	67	23.35±2.97	76	22.64±3.43	0.25
Unit 2	46	26.13±4.50	43	25.67±3.53	36	24.85±2.66	0.30
12 h post	77	24.75±2.93	72	24.84±2.77	77	24.77±3.41	0.99
Platelet Count[c]							
Baseline	78	184.14±171.76	72	215.69±132.32	80	240.29±195.31	0.12
Unit 1	72	175.39±87.28	65	211.07±138.88	74	232.94±164.91[d,e]	0.04
Unit 2	46	206.62±194.38	40	195.47±115.44	34	232.74±136.29[d]	0.58
12 h post	73	183.46±92.47	69	225.01±131.32	75	241.51±164.34[e]	0.03

[a]p-value between groups.
[b]p-value (over time) > 0.05 for all groups
[c]p-value (over time): Old LPRBCs and Young LPRBCs >0.05; CPRBCs <0.05.
[d]p<0.05 platelet count post unit 1 and post unit 2 less than baseline.
[e]p=0.04 and 0.03, respectively; CPRBC higher than LPRBC groups after unit 1 and 12 h post.

4.3 Biochemical Parameters

Free hemoglobin levels increased over transfusion time in patients who received older LPRBCs, while these levels did not change over time in patients who received younger LPRBCs or CPRBCs (Table 6). Other biochemical indices that inhibit (haptoglobin, CRP, SAP, inflammatory cytokines) or promote (2,3-DPG) O_2 delivery and RBC function did not differ between patient groups, suggesting that the biochemical effects of LPRBC and CPRBC transfusion on patients are similar.

Table 6. Comparison of Circulating Free Hemoglobin Levels (ng/mL) in Trauma Patients

Time Point	Old LPRBCs[a]		Young LPRBCs[a]		CPRBCs[a]		Overall p-value
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
Baseline	73	155 (66-329)	58	165 (93-340)	72	170 (78-306)	0.79
Unit 1	63	254 (131-491)[b]	55	245 (96-363)	66	213 (91-347)	0.24
Unit 2	34	342 (108-740)[b,c]	37	250 (132-620)	32	208 (114-438)	0.54
12 h post	75	194 (106-194)	63	201 (86-422)	69	174 (82-287)	0.53

[a]p-value (over time): Old LPRBCs = 0.01; Young LPRBCs = 0.29; CPRBCs = 0.48.
[b]p<0.05 vs. baseline.
[c]p<0.05 vs. unit 1.

In the units themselves, CPRBCs demonstrated lower levels of α2-macrogobulin, haptoglobin, CRP, SAP, and Hb (Table 7). In addition, CPRBCs had higher levels of 2,3-DPG compared to older LPRBCs.

Table 7. Comparison of Biomarkers between Units[a]

Biomarker	Old LPRBCs		Young LPRBCs		CPRBCs	
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)
α2-macroglobulin (ng/mL)	106	130 (78-251)	93	140 (85-222)	100	0.9 (0.9-1.5)[b,c]
Haptoglobin (ng/mL)	105	75 (38-130)	93	73 (37-130)	100	2.8 (0.79-3.6)[b,c]
CRP (pg/mL)	105	49 (18-162)	93	100 (30-408)[b]	100	13.5 (8.4-20.2)[b,c]
SAP (ng/mL)	105	5.5 (3.6-8.7)	93	5.6 (3.8-10.3)	100	0.06 (0.02-0.08)[b,c]
Hb (ng/mL)	114	3508 (2073-4093)	100	2427 (897-3558)[b]	108	3356 (2178-4171)[b,c]
2,3 DPG (g/L)	41	0.09 (0.02-0.17)	80	0.20 (0.8-0.37)[b]	92	0.27 (0.12-0.44)[b]

[a]Overall p-value <0.001 CPRBCs vs. Old LPRBCs and Young LPRBCs.
[b]p<0.01 vs. Old.
[c]p<0.01 vs. Young.

In study subjects, there were no differences between groups in the pro-inflammatory (IL-6, IL-8, GMCSF, INFγ, or TNFα) or the anti-inflammatory cytokines (IL-4 and IL-10) between groups or over time. The only significant difference noted was in IL-2 at 12 hours post-transfusion between the CPRBC group and the Old LPRBC group (p=0.04) (Table 8).

Table 8. IL-2 Levels (pg/mL) in Subjects

Time Point	Old LPRBCs[a]		Young LPRBCs[a]		CPRBCs[a]		p-value[b]
	n	Median (IQR)	n	Median (IQR)	n	Median (IQR)	
Baseline	78	0.10 (0-1.82)	65	0.37 (0-3.00)	71	0.72 (0-2.75)	0.14
Unit 1	71	0.13 (0-2.30)	62	0.37 (0-2.00)	70	0.58 (0-2.21)	0.21
Unit 2	40	0.40 (0-2.27)	36	0.30 (0-2.70)	32	0.62 (0-1.42)	0.65
12 h post	74	0.14 (0-1.62)	63	0.35 (0-2.75)	68	1.05 (0-2.83)[c]	0.04

[a]p-value (over time): Old LPRBCs = 0.98; Young LPRBCs = 0.91; CPRBCs = 0.57.
[b]p-value between groups.
[c]CPRBC > Old LPRBC at 12 h post.

Cytokine levels were measured in each of the units transfused (Table 9). IL-4, IL-6, IL-10, GMCSF, and INFγ were elevated in CPRBC units compared to Old LPRBC units. Additionally, IL-4, IL10, GMCSF, and INFγ were elevated in the CPRBC units compared to Young LPRBC units.

Table 9. Cytokine Levels (pg/mL) in PRBC Units

Cytokine	Old LPRBCs n	Old LPRBCs Median (IQR)	Young LPRBCs n	Young LPRBCs Median (IQR)	CPRBCs n	CPRBCs Median (IQR)	Overall p-value
IL-2	106	0 (0-0)	89	0 (0-0)	92	0 (0-0)	0.479
IL-4	106	0 (0-0)	89	0 (0-0)	92	0.5 (0.3-0.8)[a,b]	<0.001
IL-6	106	0 (0-0)	89	0 (0-0.2)	92	0 (0-0.2)[a]	0.014
IL-8	106	0.86 (0-2.73)	89	1.24 (0.01-2.87)	92	1.49 (0.47-2.52)	0.195
IL-10	106	0 (0-0)	89	0 (0-0.1)	92	1.3 (0.7-1.6)[a,b]	<0.001
GMCSF	106	30 (9-67)	89	25 (10-59)	92	103 (78-163)[a,b]	<0.001
INFγ	106	0 (0-0)	89	0 (0-0)	92	0 (0-0)[a,b]	0.002
TNFα	106	0 (0-0)	89	0 (0-1)	92	0 (0-1)	0.211

[a]p<0.01 vs. Old.
[b]p<0.01 vs. Young.

3.4 Clinical Outcomes

In line with our biochemical and coagulation data, clinical adverse outcomes did not differ between groups (Table 10).

Table 10. Comparison of Adverse Clinical Outcomes between Groups

Adverse Clinical Outcomes	Percent Occurrence of-- Old LPRBCs (n=85)	Young LPRBCs (n=82)	CPRBCs (n=86)	Overall p-value
Acute Renal Failure	8	9	12	0.45
Respiratory Failure	19	15	24	0.46
Liver Failure	1	0	0	0.22
Acute Respiratory Distress Syndrome	2	6	5	0.46
Ventilator Associated Pneumonia	11	13	16	0.28
Infection	26	30	28	0.77
Sepsis	7	6	9	0.58
Deep Vein Thrombosis	15	17	15	0.97
Pulmonary Embolism	7	4	6	0.73
Stroke	1	0	0	0.22
Mortality	3	4	4	0.65

5.0 DISCUSSION

5.1 Key Research Accomplishments

1. Transfusion of Old LPRBCs resulted in a reduction in tissue oxygenation.
2. Transfusion of Young LPRBCs did not affect tissue oxygenation.
3. Transfusion of CPRBCs resulted in an increase in tissue oxygenation.
4. Despite starting with the same hematocrit, patients who received CPRBCs required two fewer units of blood.
5. There were elevated free hemoglobin levels in the Old LPRBC group after transfusion of each unit.
6. Transfusion of CPRBCs resulted in an increase in the counter-inflammatory cytokines IL-4 and IL-10.
7. Potentially harmful biochemical markers were lower in the CPRBC units.

5.2 Reportable Outcomes

Taken together, these data support our current hypothesis that the transfusion of CPRBCs is a safe practice and results in equivalent clinical outcomes compared to patients who receive LPRBCs. It is important to remember that patients in our preliminary studies were relatively stable and generally only required one to two units of blood. Clinical differences between groups may occur in a more critically ill patient population that is receiving larger RBC volumes.

Additionally, as with our single center trial, the biochemical profile of CPRBCs is profoundly superior to LPRBCs, characterized by lower levels of α2-macrogobulin, haptoglobin, CRP, SAP, and Hb. CPRBCs have higher levels of 2,3-DPG compared to older LPRBCs, which should improve their ability to deliver O_2 to underperfused tissues. These results support the increased use of frozen deglycerolized red blood cells, both in theater and in the continental United States due to their logistical advantages, avoidance of the storage lesion, improved oxygen delivery, and their safety profile.

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LIST OF ABBREVIATIONS AND ACRONYMS

2,3-DPG	2,3-diphosphogycerate
AUC	area under the curve
CPRBC	cryopreserved packed red blood cells
CRP	C-reactive protein
GMCSF	granulocyte-macrophage colony-stimulating factor
Hb	hemoglobin
ICU	intensive care unit
IL	interleukin
INFγ	interferon gamma
ISS	Injury Severity Score
IQR	interquartile range
LPRBC	liquid preserved red blood cells
NO	nitric oxide
PRBC	packed red blood cells
RBC	red blood cells
SAP	serum amyloid P
SNO-Hb	S-nitrosohemoglobin
SD	standard deviation
TEG	thrombelastography
TNFα	tumor necrosis factor alpha
StO_2	tissue oxygenation
WBC	white blood cells